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Cryobiology 52 (2006) 48–61
CRYOBIOLOGY
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Plant vitrification solution 2 lowers water content and alters freezing behavior in shoot tips during cryoprotection ☆

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Received 5 July 2005; accepted 21 September 2005
Available online 29 November 2005

Abstract

Plant shoot tips do not survive exposure to liquid nitrogen temperatures without cryoprotective treatments. Some cryoprotectant solutions, such as plant vitrification solution 2 (PVS2), dehydrate cells and decrease lethal ice formation, but the extent of dehydration and the effect on water freezing properties are not known. We examined the effect of a PVS2 cryoprotection protocol on the water content and phase behavior of mint and garlic shoot tips using differential scanning calorimetry. The temperature and enthalpy of water melting transitions in unprotected and recovering shoot tips were comparable to dilute aqueous solutions. Exposure to PVS2 changed the behavior of water in shoot tips: enthalpy of melting transitions decreased to about 40 J g H_2O^{-1} (compared to 333 J g H_2O^{-1} for pure H_2O), amount of unfrozen water increased to $\sim$ 0.7 g H_2O g dry mass^{-1} (compared to $\sim$0.4 g H_2O g dry mass^{-1} for unprotected shoot tips), and a glass transition (T_g) at −115 °C was apparent. Evaporative drying at room temperature was slower in PVS2-treated shoot tips compared to shoot tips receiving no cryoprotection treatments. We quantified the extent that ethylene glycol and dimethyl sulfoxide components permeate into shoot tips and replace some of the water. Since T_g in PVS2-treated shoot tips occurs at −115 °C, mechanisms other than glass formation prevent freezing at temperatures between 0 and −115 °C. Protection is likely a result of controlled dehydration or altered thermal properties of intracellular water. A comparison of thermodynamic measurements for cryoprotection solutions in diverse plant systems will identify efficacy among cryopreservation protocols.
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Keywords: Cryopreservation; *Allium sativum*; Garlic; *Mentha*; Mint; Shoot tip; Differential scanning calorimetry

Prevention of intracellular ice formation and growth during cooling, storage, and thawing is critical for successful cryopreservation. This is most simply accomplished by removing water that would otherwise form lethal ice crystals within cells during cooling to liquid nitrogen (LN) temperatures. Many cryopreservation protocols reduce water content either by drying desiccation tolerant tissues or by treating shoot tips with concentrated solutions which osmotically remove water from cells. Successful cryopreservation protocols balance cell water content so that both freezing injury and desiccation damage are minimized

☆ This work was funded by institutional sources. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

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0011-2240/$ - see front matter. Published by Elsevier Inc.
doi:10.1016/j.cryobiol.2005.09.004

[15,27,36,46]. Drying cells, either by exposure to air, concentrated solutions or freeze dehydration, does not provide adequate protection from cryogenic temperatures for most non-hardy cell types. Bathing cells in solutions containing specific molecules provided a major breakthrough in modern cryobiology (e.g., [16,34]) and the discovery that some cryoprotectants are naturally produced by cold-hardy plants during winter [21] provided clear evidence that cryoprotection requires more than just adjusting water content.

Since the discovery that cells treated with specific cryoprotecting solutions survive exposure to cryogenic temperatures, variations on solution composition have been developed for plant cells [24,33,37,42,43]. These solutions are called "vitrification" or "vitrifying" solutions to emphasize their role in preventing intracellular ice by promoting glass formation in the aqueous domain of cells [12,16,35,37]. Recent concepts of aqueous glasses describe the complexity of temperature responses in these supercooled, super-viscous solutions (e.g., [1,51] and references therein). Glass transitions occur at about −115 °C in PVS2, the cryoprotectant solution most commonly used for plants [37]. Despite their name and routine usage, the mechanism by which vitrification solutions protect cells from damage is poorly understood [20]. They may function as elaborate desiccants and decrease the amount of water that is available to form lethal ice crystals [38]. Alternatively, they may stabilize cell structures during desiccation and cooling as described by literature on glassy states in dry biological systems [6,11]. Finally, vitrification agents may structure water remaining in cells so that it is less likely to freeze [54]. Cryoprotectant solutions, especially those containing glycerol, are toxic to cells and prolonged exposure can be problematic [14,17,50].

Thermal analyses of plant materials using differential scanning calorimetry (DSC) demonstrate a strong relationship between the presence of water freezing or melting transitions and damage at subfreezing temperatures [4,12,13,23,25,26,39,44–47,52]. Evidence of first order water transitions (i.e., freezing and melting) are observed in seeds (e.g., [44–46,52,18]), pollen [8], winter-hardy buds [47], naked shoot tips of olive [25], and shoot tips encapsulated in calcium alginate beads [4,13,25,39] that contain more than 0.25–0.4 g H_2O g dm^{-1} (dm; dry mass). Plant organs that survive LN temperatures usually contain this amount of water or less (e.g., [12,46,52]), though the window of acceptable water contents can be widened by increasing cooling rate to over 100 °C s^{-1} [52,53].

Water at water contents less than 0.25 g H_2O g dm^{-1} is often referred to as 'unfreezable' or 'unfrozen' to indicate that motional restrictions are too great to allow the molecular reorganizations necessary for crystallization within a practical time frame (the cooling rate in a typical DSC experiment is usually 1–20 °C min^{-1}). With such restricted mobility, water at these low water contents will vitrify with further cooling, if it has not already vitrified at room temperature, and second order transitions, representing glass formation, may be detected. Second order transitions (heat capacity changes) in seeds (e.g., [51]) are small and broad compared to those observed for simpler solutions and alginate beads (e.g., [5,25]). DSC measurements of water in biological materials may be confounded by the presence of interfering signals from lipids and by drying, cooling and warming protocols (e.g., [8,13,39,51]). Devitrification and recrystallization, observed as an exothermic event in warming DSC scans, are sometimes evident in materials containing 0.2–0.5 g H_2O g dm^{-1} [13,45] or hydrated materials treated with cryoprotectants [4,32,37], and portend a risk of damaging ice formation if cryoexposed materials are warmed as slowly as they are in DSC experiments [27,35,37]. Recrystallization does not reflect the stability of the glass per se [4,13,39], but rather the mobility within the fluid non-vitrified material. In addition to faster warming, recrystallization can be lessened by increased drying [4,7,13,45] or longer incubation in cryoprotectant solutions [23], though both procedures can damage cells in other ways.

An evaluation of the first and second order transitions and water contents of cryoprotected shoot tips will reveal potential mechanisms by which PVS2 is effective. Comparisons of the thermal properties of treated biological materials and cryoprotectant solutions provide valuable insights towards understanding the mechanism of cryoprotection [3,5,15]. We have used DSC to measure the size and temperature of first (exothermic and endothermic events) and second order transitions (glass transitions) within treated shoot tips of garlic and mint at each step of a cryopreservation procedure that uses PVS2 as the cryoprotectant. Bathing garlic shoot tips in cryoprotectant solutions reduces the size and sometimes the temperature of first order transitions [23], but it is unclear whether freezing is reduced because the cells dried out in response to the cryoprotectants or because the cryoprotectants altered the

thermodynamic properties of the water. Our study combines detailed measurements of first and second order transitions detected using DSC with assessments of cryoprotectant penetration and cell water content to show that cryoprotectant solutions replace some water in cells and alter the freezing behavior of the remaining water. This information can be used to determine treatment solutions and exposure times that improve survival at LN temperatures.

Materials and methods

Plant materials

Summer harvested bulbs of garlic cultivar "German Extra Hardy," a porcelain type [48], were fully cured and stored in paper bags within cardboard boxes at 22 °C until they were used between the following September and January. A single shoot tip ($1.5 \times 1.5 \times 1.5$ mm, containing at least 0.5 mm of basal plate) was excised from each clove, placed onto garlic medium [49], and kept at 5 °C for 48 h.

Mentha x piperita L. cultivar "Todd's Mitcham Peppermint" (PI 557973) plants were maintained in vitro and propagated on mint medium (MS basal mineral and vitamin medium [30] with 3% sucrose and 1% agar). Shoot tips ($0.5 \times 0.5 \times 1$ mm) were excised from lateral buds that emerged from excised nodal sections cultured on mint medium for 3 days. Shoot tips were placed overnight in liquid 0.3 M sucrose with half-strength MS.

Cryopreservation

Excised shoot tips were immersed in 2 M glycerol containing 0.6 M sucrose in half-strength MS salts (glycerol + sucrose) for 20 min at 25 °C. Shoot tips were submerged in PVS2 (15% w/v ethylene glycol, 15% w/v DMSO, 30% w/v glycerol, and 13.7% w/v sucrose in MS salts) at 0 °C for 5–60 min [38]. For garlic shoot tips, three drops of PVS2 solution, each containing a shoot tip, were placed on aluminum foil strips and immersed in vacuum-treated liquid nitrogen (LN slush, −210 °C). Shoot tips on foils were warmed by immersing in 1.2 M sucrose and held for 20 min at 25 °C. For mint, 10 shoot tips were placed in a 1.2 ml cryovial containing 0.5 ml PVS2 and plunged into liquid nitrogen. Vials were warmed at 40 °C for 2 min, then shoot tips were diluted into 1.2 M sucrose for 20 min at 25 °C. Shoot tips were subsequently cultured on solid medium. These cryopreservation protocols are used routinely in our laboratory to preserve garlic [49] and mint [42] germ plasm.

Water and cryoprotectant content

Standard gravimetric comparisons of fresh and dry mass cannot be used to measure water contents in cryoprotected shoot tips because some cryoprotectants are also volatile. Steady state measurements of shoot tip mass were achieved after heating shoot tips at 90 °C for 24 h. We assigned this value as the dry mass of the tissues plus the mass of non-volatilized cryoprotectant. The proportion of water in the volatile component of the sample was measured in subsets of shoot tips after each cryoprotection step by Karl Fischer titration [29] using Karl Fischer reagent (Fisher Chemicals, Fair Lawn, NJ) and the manufacturer's directions. Based on the Bunsen reaction, Karl Fischer titrations specifically detect water, which is consumed when an alcohol and amine oxidize in the presence of iodine. The endpoint is detected by a color or voltage change.

The amount of volatilized cryoprotectant was then determined from the difference between total volatile mass and the fraction that could be attributed to water. The mass of volatilized cryoprotectant was added back to the dry mass measured from oven dry weights to give a corrected dry mass that was used for DSC calculations and data analysis. Water content of shoot tips was manipulated by air drying on the benchtop (relative humidity ~30%) or exposure to PVS2 for 5–120 min. Time course data for cryoprotectant volatilization at room temperature were fitted to logarithmic curves ($r^2 > 0.93$) in order to estimate the proportion of cryoprotectants in the tissues as a function of air drying time or time exposed to PVS2.

Differential scanning calorimetry

Shoot tips of garlic (1 tip per sample) and mint (8 tips per sample) were blotted dry, hermetically sealed into Perkin-Elmer (Norwalk, CY, USA) volatile aluminum pans (20 μl capacity), weighed, and subjected to calorimetric measurements within 5 min. Thermal behavior of apical shoot tips was measured after each step of the cryopreservation procedure using a Perkin-Elmer differential scanning calorimeter (DSC7) (Norwalk, CT, USA), calibrated for temperature with methylene chloride (−95 °C) and indium (156.6 °C) and for energy with indium (28.54 J g^{-1}) [18]. Samples were cooled from

20 °C to −150 °C and rewarmed to 20 °C at 10 °C min^{-1}. Temperatures and energies of first and second order transitions were calculated from heat flow data using Perkin-Elmer software. Onset temperatures for ice crystallization and melting were calculated from the intersection of the baseline and the tangent to the steepest part of the transition peak, and glass melting transitions were calculated from the midpoint of the discontinuity in the baseline of the scan. Enthalpy of the ice melting was calculated from the area encompassed by the transition peaks and a projected linear baseline. Heat capacity of the glass transition was calculated from the power of the baseline shift. The melting enthalpy per mass of water and the amount of water remaining unfrozen in tissues was calculated from the slope and x-intercept, respectively, of the linear regression between water content and the melting enthalpy per dry mass of the shoot tips, where water content was manipulated by air drying or exposure time to PVS2 (see [18] for details of analyses). Calorimetric data were collected from three to eight replicates per treatment.

The effects of solutions were separated from the effects of LN exposure by measuring water content and thermal behavior in mint shoot tip controls that had undergone cryoprotection and recovery treatments. Mint shoot tips were used to test the effect of cooling rate on DSC warming profiles. Shoot tips sequentially treated with glycerol + sucrose and then PVS2 (30 min) were sealed into a DSC pan and plunged into LN. Pans were subsequently cold-loaded into a pre-chilled DSC (−150 °C) for analysis. A limited supply of bulbs precluded conducting this experiment with garlic.

Results

Calorimetric measurements and contents of water and cryoprotectants were assessed in garlic and mint shoot tips at each step in the cryopreservation process to determine how cryoprotection by PVS2 reduced intracellular ice formation and the rate at which this protection was lost during thawing and recovery. Regrowth percentages of mint shoot tips exposed to PVS2 at 0 °C for 30 min were 95 ± 5 and 75 ± 8% before and after LN exposure, respectively [50], and regrowth percentages of garlic shoot tips were 82 ± 11 and 35 ± 10% for the analogous treatments (Volk, unpublished). Lethality was not apparent until after 7 days of recovery for mint and 14 days of recovery for garlic, and so it is likely that a mixture of killed and living samples were analyzed by DSC after PVS2, LN, 1.2 M sucrose, and recovery treatments.

Substantial water freezing and melting transitions were observed in DSC scans of garlic (Figs. 1A and B) and mint (Figs. 1C and D) shoot tips before PVS2 was applied and after the 1.2 M sucrose recovery treatment which presumably dilutes PVS2 within cells. The temperature of the freezing transition decreased from about −15 °C (both species) to about −32 °C (both species) after exposure of shoot tips to glycerol + sucrose (scan #2, Figs. 1A and C) and increased again during recovery from about −30 °C immediately after thawing in 1.2 M sucrose to about −15 °C after 5 days recovery (compare scans #5 and #7 in Figs. 1A and C and 2B). Similar temperature changes were observed for melting transitions (Figs. 1B and D) and are summarized in Fig. 2A. Shoot tips of both mint and garlic had the highest melting temperatures, between −3 and −8 °C, after the initial culture on medium or in 0.3 M sucrose, and after 1 or 5 days recovery treatments. Treatment with glycerol + sucrose lowered the melting temperature to about −20 °C in both garlic and mint, and PVS2 exposures of 5–15 min reduced the melting temperatures to −50 °C in shoot tips of garlic that still exhibited melting transitions during warming.

The size of the melting transitions changed dramatically during cryoprotection and recovery, with the largest peaks observed in shoot tips after 1 and 5 days of recovery (Figs. 1B and D; full scale of ordinate extends to 120 mW mg dm^{-1}, but is not shown to show finer details in more dehydrated samples; see also Fig. 2C). Treating shoot tips with glycerol + sucrose decreased the size of the endothermic peaks observed upon warming (compare curves 1 and 2 in Figs. 1B and D; see also Fig. 2C). Exposure to PVS2 at 0 °C progressively decreased the size of melting transitions so that they were not observable within 5 and 30 min of treatment for mint and garlic, respectively (Figs. 2 and 3).

The dehydrating and rehydrating effect of the cryoprotection and recovery procedures can also be visualized by measurements of the water content in the shoot tips (Fig. 2D). Water content for garlic and mint shoot tips from medium and 0.3 M sucrose, respectively, is about 4 g H_2O g dm^{-1}. Treatment with glycerol + sucrose lowers the water content to about 2 g H_2O g dm^{-1}, and exposure to PVS2 lowers the water content further. Water contents

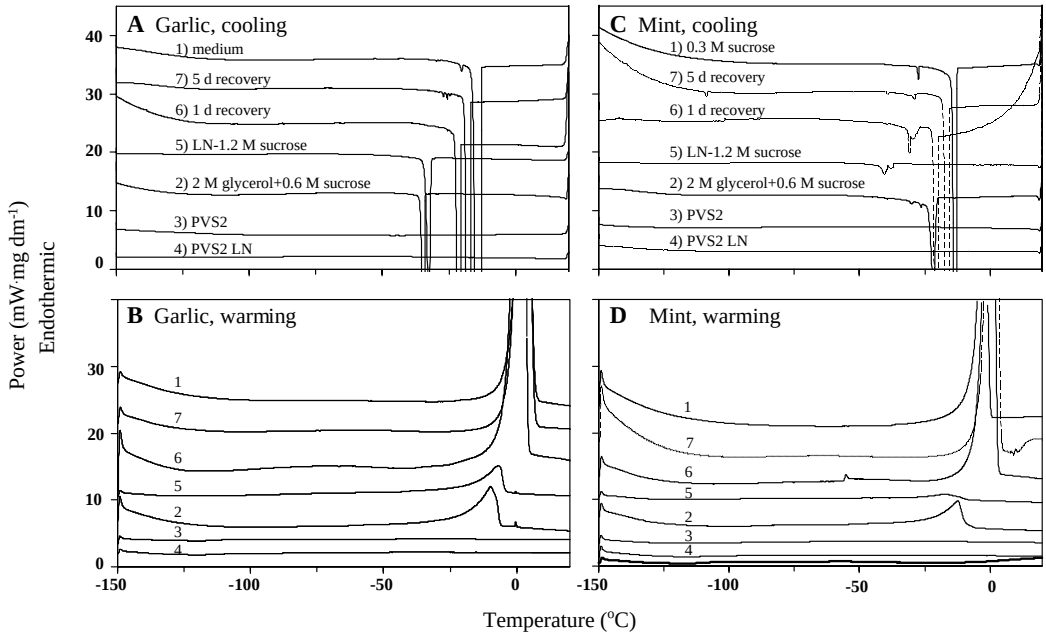

Fig. 1. DSC scans showing first order transitions that occur during garlic cooling (A) and warming (B) and mint cooling (C) and warming (D). Single garlic or eight mint shoot tips were processed through the cryoprotection protocol to the indicated step of the procedure and subsequently loaded into the DSC: (1) Medium (or liquid 0.3 M sucrose), 24 h, 25 °C, (2) 2 M glycerol + 0.6 M sucrose, 20 min, 25 °C, (3) PVS2, 30 min, 0 °C, (4) PVS2, 30 min 0 °C, LN, (5) LN-1.2 M sucrose, 20 min, 25 °C, (6) 1 day recovery on media, and (7) 5 days recovery on media. Bold line in Fig. 1D represents PVS2 and LN-treated mint shoot tips that were plunged into LN and then loaded into the DSC sample holder precooled to −150 °C before warming.

increased to 1–2 g H_2O g dm^{-1} when shoot tips were thawed in 1.2 M sucrose.

Water contents increased when recovering shoot tips were placed back on solid medium (Fig. 2D). Water contents of 4.4 ± 0.2 and 6.2 ± 0.2 g H_2O g dm^{-1} were measured in untreated garlic and mint shoot tips (i.e., shoot tips that were grown on solid medium and were not exposed to 0.3 M sucrose, glycerol + sucrose solution, PVS2 and LN) (data for garlic in Fig. 2D; data for mint not shown). Mint samples that received cryoprotection treatments but were not exposed to LN readjusted to initial water contents within 1 day and stabilized within 5 days (water contents were 5.7 ± 0.3 and 6.1 ± 0.1 g H_2O g dm^{-1} for 1 and 5 days post-recovery, respectively) (data not shown). In contrast, water contents for LN-exposed shoot tips increased to higher levels (10.3 ± 0.1 and 7.5 ± 0.5 g H_2O g dm^{-1} for garlic and mint, respectively) after 1 day recovery and resumed levels comparable to initial measurements after 5 days.

Melting events were progressively smaller in garlic shoot tips as time in PVS2 at 0 °C increased from 5 to 30 min (Fig. 3). A recrystallization event, seen as an exothermic peak during warming, sometimes occurred in garlic shoot tips after 5 or 15 min PVS2 exposures at 0 °C (Fig. 3, open arrows). After a 5 min PVS2 exposure at 0 °C, garlic and mint shoot tip water contents varied between 1.61 and 2.34 g H_2O g dm^{-1} and 0.70 and 0.96 g H_2O g dm^{-1}, respectively (Fig. 3). Both mint and garlic shoot tips exposed to PVS2 for more than 30 min at 0 °C exhibited only second order glass transitions during cooling and warming (Figs. 2–4). Both glass and melting transitions were observed in mint shoot tips treated with glycerol + sucrose, and the midpoint temperature of the glass transition decreased from −80 to −115 °C after treatment with PVS2 for 5 min (Fig. 4A). The midpoint temperature of the glass transition remained at −115 °C for both garlic and mint shoot tips regardless of the time in PVS2 (Fig. 4A). The size of the baseline shift at −115 °C decreased with exposure time to PVS2 in garlic shoot tips (Fig. 4B).

DSC warming thermograms of mint shoot tips treated with PVS2 for 15 min were similar among samples cooled at 10 °C min^{-1} or rapidly cooled by plunging into liquid nitrogen and then loaded into a DSC chamber precooled to −150 °C. Both cooling methods gave glass transition temperatures of

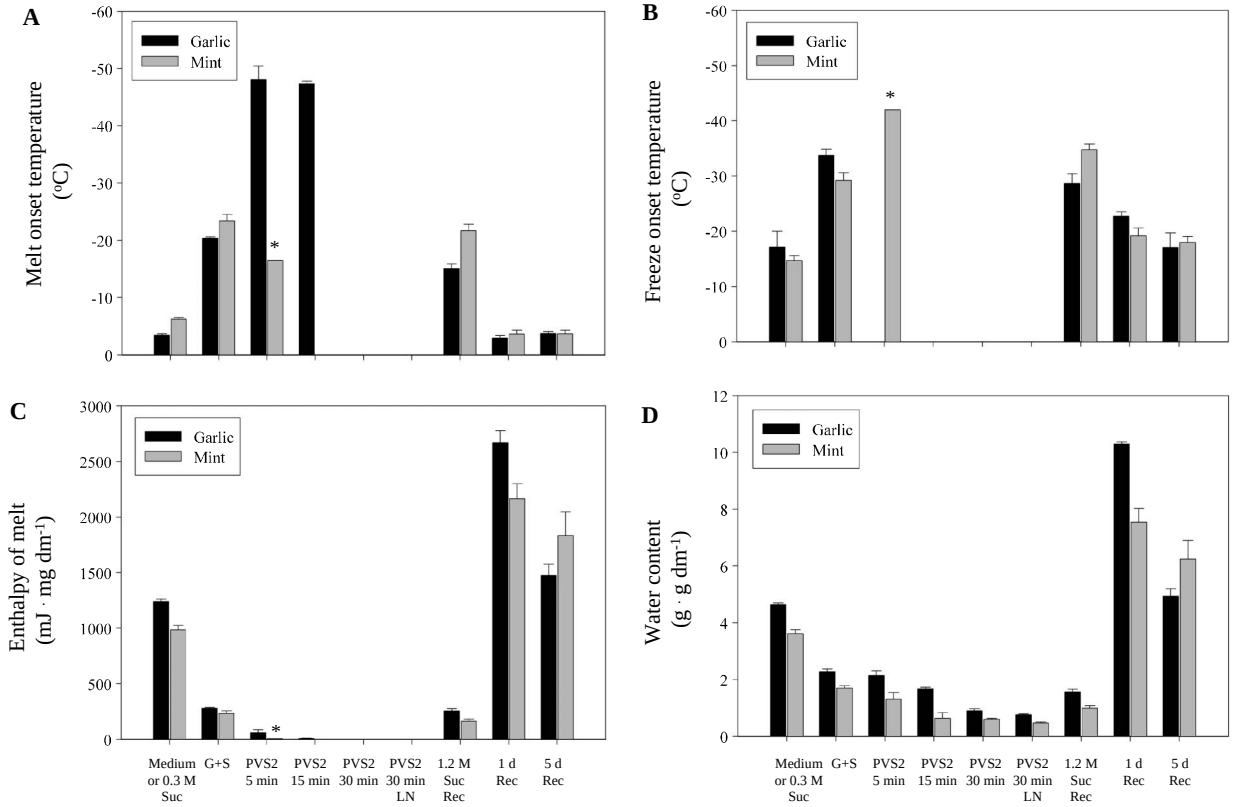

Fig. 2. Melting (A) and freezing (B) onset temperature and melting enthalpy (C) of first order transitions that occur upon warming in garlic and mint shoot tips, as measured using DSC. Water contents (dry mass basis) (D) of shoot tips after each step of the cryoprotection procedure (as shown in order across the *x* axis): Medium (or liquid 0.3 M sucrose), 24 h, 25 °C; 2 M glycerol + 0.6 M sucrose, 20 min, 25 °C; PVS2, 5 min, 0 °C; PVS2, 15 min, 0 °C; PVS2, 30 min 0 °C; PVS2, 30 min 0 °C, LN; LN-1.2 M sucrose, 20 min, 25 °C; 1 day recovery on media; 5 days recovery on media. Bars represent average ± SE of three to eight replicate samples. Temperatures for shoot tips treated with PVS2 for ⩾15 min are not given because melting transitions were not observed. The asterisks indicate data for the single sample out of eight replicates exposed to PVS2 for 5 min that showed freezing and melting transitions.

−115 °C and no melting transitions (Fig. 1D compare scan #4 with bold scan below it). Thus, for fully protected shoot tips, the normal DSC protocol of cooling at 10 °C min^{-1} provided thermal information for mint shoot tips treated for 30 min with PVS2 similar to the more rapid cooling treatment of plunging DSC pans into LN, which was comparable to the cryopreservation protocols.

PVS2 components replace cell water within shoot tips

Changes in water content, dry mass, and content of cryoprotectant molecules that volatilize during heating to 90 °C (e.g., ethylene glycol and dimethyl sulfoxide) were determined for mint and garlic shoot tips after incubation in PVS2 (Fig. 5). About 0.6 mg of water was removed from a single garlic shoot tip that had been treated with glycerol + sucrose and exposed to PVS2 for 120 min; 0.4 mg of water were removed from a set of eight mint shoot tips exposed to PVS2 for 30 min (Figs. 5A and C). Despite the loss of water, the fresh mass of the shoot tips did not change appreciably because of the compensating effect of penetrating cryoprotectants. In garlic shoot tips, cryoprotectant content equilibrated to about 1 mg cryoprotectant mg dm^{-1} after 60 min of exposure to PVS2 (Fig. 5B). For mint, cryoprotectant levels equilibrated to about 1.5 mg cryoprotectant mg dm^{-1} within 15–30 min of PVS2 exposure (Fig. 5D). Water contents decreased to about 0.6 g H_2O g dm^{-1} within 30 and 60 min for mint and garlic shoot tips, respectively, and then decreased to about 0.4 g H_2O g dm^{-1} after an additional 30 min exposure period (Figs. 5B and D). The different sizes of garlic and mint explants may account for the differences in the kinetics of dehydration and cryoprotectant permeation.

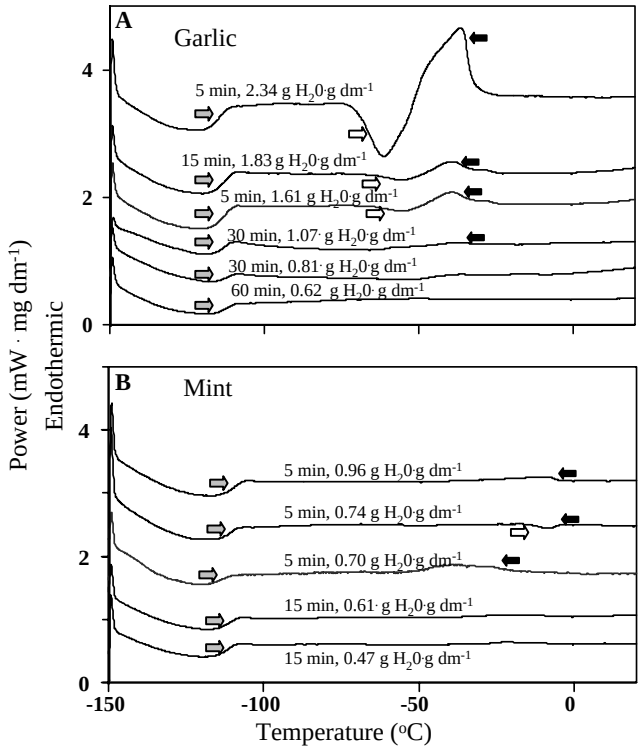

Fig. 3. DSC scans showing first order transitions that occur during warming as single garlic (A) and sets of eight mint (B) shoot tips are treated with PVS2 for between 5 and 60 min. Length of PVS2 exposure and water content values are provided for each scan. Gray arrows denote second order transitions, white arrows denote first order freezing transitions upon warming (recrystallization) and black arrows denote first order melting events.

PVS2 components change the freezing and melting behavior of water remaining in cells

DSC measurements of shoot tips during the cryoprotection process show that first order transitions representing phase changes in water are lost, and second order glass formation transitions at about −115 °C appear in shoot tips exposed to PVS2 (Figs. 1–4). To test the hypothesis that the observed changes were a simple consequence of dehydration, we constructed a series of DSC scans of shoot tips treated with culture medium (or 0.3 M sucrose), 2 M glycerol + 0.6 M sucrose, and/or PVS2 and then air-dried to different water contents. We hypothesized that if the observed changes in thermal behavior were a direct effect of dehydration alone, then the amount of unfrozen water and the enthalpy of water that froze, expressed per mass of water rather than per dry mass of tissue, would be similar among treatments (see [18] for details of the analyses). The enthalpy of water melting transitions (determined from the slope of regression between enthalpy and water content (Fig. 6, Table 1)) was 309 ± 11 J g H_2O^{-1} for garlic shoot tips on medium and 317 ± 18 J g H_2O^{-1} for mint shoot tips in 0.3 M sucrose, which is comparable to the enthalpy of pure water (333 J g H_2O^{-1}). Treatment with glycerol + sucrose decreased the melting enthalpy on a per mass of water basis to 162 ± 16 and 231 ± 30 J g H_2O^{-1} for garlic and mint, respectively. Treatment with PVS2 decreased the melting enthalpy further to near 40 J g H_2O^{-1} (Fig. 6, Table 1). Furthermore, the unfrozen water content (x-intercepts in Fig. 6 and Table 1) progressively increased with each cryoprotection step from 0.36 to 0.49 to 0.74 g H_2O g dm^{-1} for garlic shoot tips that were untreated, exposed to glycerol + sucrose and then to PVS2, and from 0.45, 0.67, and 0.68 g H_2O g dm^{-1} for mint shoot tips after the same series of treatments.

The change in water behavior can be graphically depicted by DSC scans of garlic and mint shoot tips that were exposed to medium or 0.3 M sucrose, glycerol + sucrose, or PVS2 and then adjusted to similar water contents by air drying (Fig. 7). Freezing and melting transitions are evident at about

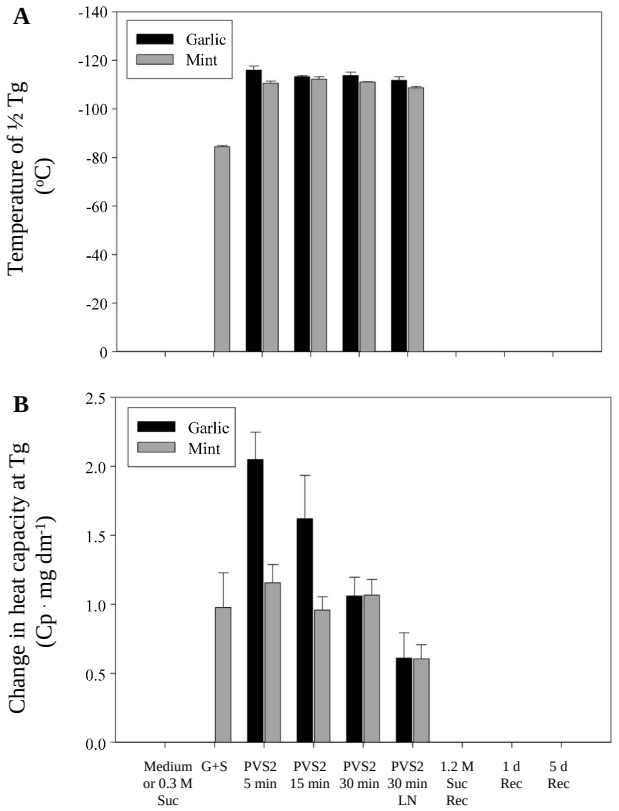

Fig. 4. Second order transition temperatures (A) and magnitude (heat capacity) (B) that occur upon warming in single garlic and sets of eight mint shoot tips, as identified using DSC. Treatments are the same as those shown in Fig. 2. Bars represent average ± SE of three to eight replicate samples.

−25 °C in shoot tips receiving no cryoprotecting treatments that were air dried to about 0.78 g H_2O g dm^{-1} for garlic on medium (Figs. 7A and B) and 0.82 g H_2O g dm^{-1} for mint in 0.3 M sucrose (Figs. 7C and D). The size of the transition peaks is smaller in shoot tips that were exposed to glycerol + sucrose and then adjusted to a similar water content. There is no evidence of freezing and melting events in PVS2-treated shoot tips adjusted to similar water contents as the previous steps, although a glass transition is detected at −115 °C.

PVS2 components impede water loss during air drying

Experiments described in this paper show that cryoprotectants dehydrate plant cells and lower the propensity of the remaining water molecules to freeze. The latter effect suggests that the residual water may have restricted mobility, and we hypothesized that this may also affect the rate that shoot tips equilibrate to ambient relative humidity (30% RH at 22 °C). Drying time courses show that equilibrium water contents were achieved most rapidly, within about 3 h of air drying, for shoot tips cultured in medium or 0.3 M sucrose, despite the initially high water content described in Fig. 2D (Fig. 8). Water contents for garlic shoot tips treated with glycerol + sucrose or glycerol + sucrose and then PVS2 remained above 0.20 g H_2O g dm^{-1} for about 16 h (Fig. 8A) and equilibrated to 0.08 g H_2O g dm^{-1} after about 2 weeks (data not shown). In contrast, water contents greater than 0.5 g H_2O g dm^{-1} were maintained for about 18 h in mint shoot tips exposed to glycerol + sucrose (Fig. 8B), but eventually decreased to about 0.2 g H_2O g dm^{-1} within 2 months (data not shown). PVS2 treated shoot tips of mint reached the equilibrium water content of 0.08 g H_2O g dm^{-1} in 3 days.

Discussion

We provide experimental evidence supporting three mechanisms by which PVS2 aids cryoprotection

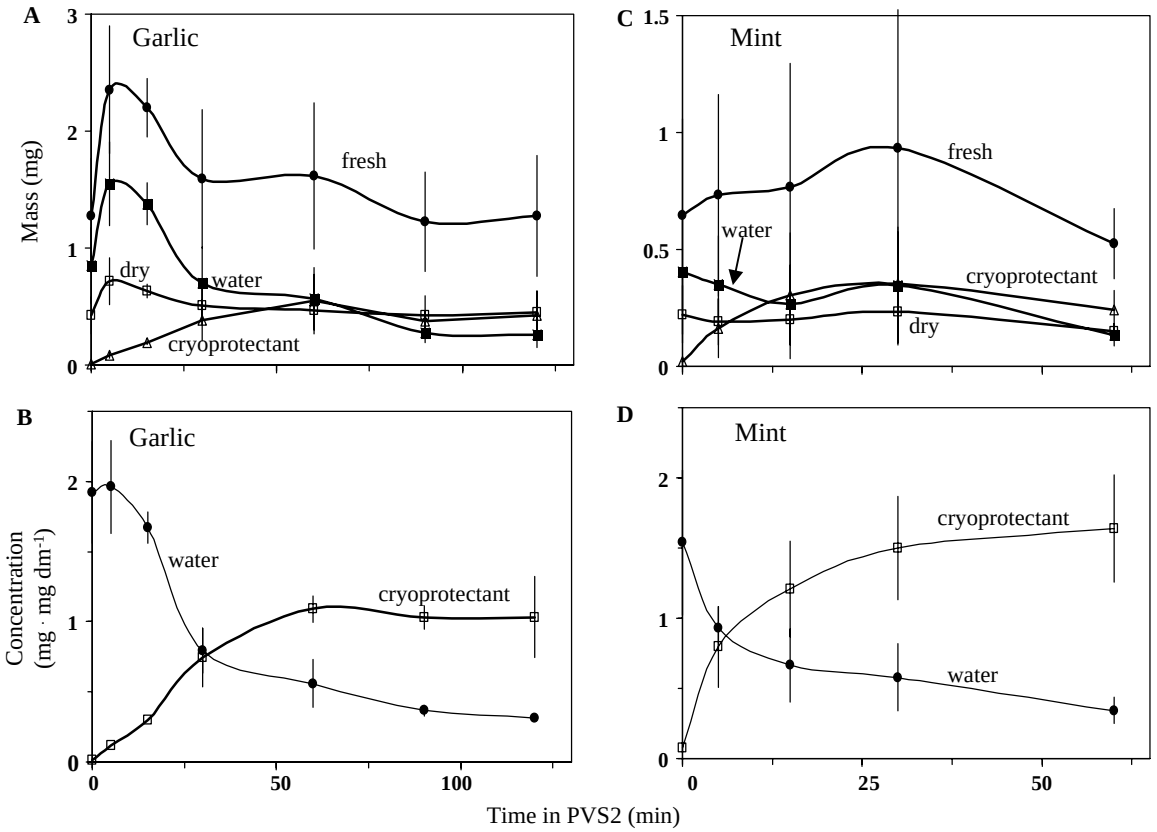

Fig. 5. Masses of fresh shoot tips, water, dry shoot tips, and accumulated volatile components of the cryoprotectant solution measured in single garlic shoot tips (A) and sets of eight mint shoot tips (C). Concentrations of water and cryoprotectants in single garlic shoot tips (B) and sets of eight mint shoot tips (D) after various lengths of PVS2 exposure (0 °C).

of shoot tips: PVS2 (1) replaces cellular water, (2) changes freezing behavior of water remaining in cells, and (3) impedes water loss during air drying. Because solution components permeate into cells, PVS2 treatment can broaden allowable water contents in cryopreserved shoot tips by lessening the damage from excessive cell shrinkage and limiting the risk of ice formation and growth (Fig. 5). These effects were observed in both garlic and mint shoot tips, despite the differences in the shoot tip size of the two species. PVS2 presence was apparent in mint shoot tips (ca. 0.1 mg fresh mass per shoot tip) within 5 min of exposure at 0 °C, while equivalent effects were observed in the larger garlic shoot tips (ca. 1.5 mg per shoot tip) after 30 min. The consistency of the observed effects of PVS2 in two diverse explant sources suggests that our observations may be generally applicable across a broad range of species.

Penetration of some components of PVS2 into the cytoplasm is key to its cryoprotective function. While sometimes considered impermeable (e.g., [36]), plant cell walls and membranes appear to be permeable to dimethyl sulfoxide and ethylene glycol [2,3,24,31]. Toxicity studies, which show glycerol-induced damage exacerbated by dimethyl sulfoxide and ethylene glycol [40,50], also point to the permeability of cells to components of PVS2. The kinetics of PVS2 entry into cells (Fig. 5) is consistent with a previous report of dimethyl sulfoxide accumulation in garlic shoot tips, as measured by HPLC [22]. The mass of cryoprotectant entering shoot tips is commensurate with the mass of water lost from the tissues (Fig. 5). Replacement of water by non-aqueous components occurs (e.g., dry matter accumulation during plant embryogenesis), and is likely essential for preventing damage from loss of critical cell volume [28] or surface area [41]. The mechanisms which allow cell dehydration without cell shrinkage remain unclear.

Dehydration of shoot tips as a result of exposure to PVS2 had some expected and unexpected effects on the phase behavior of water. As expected, the size of first order transitions (freezing and melting) was

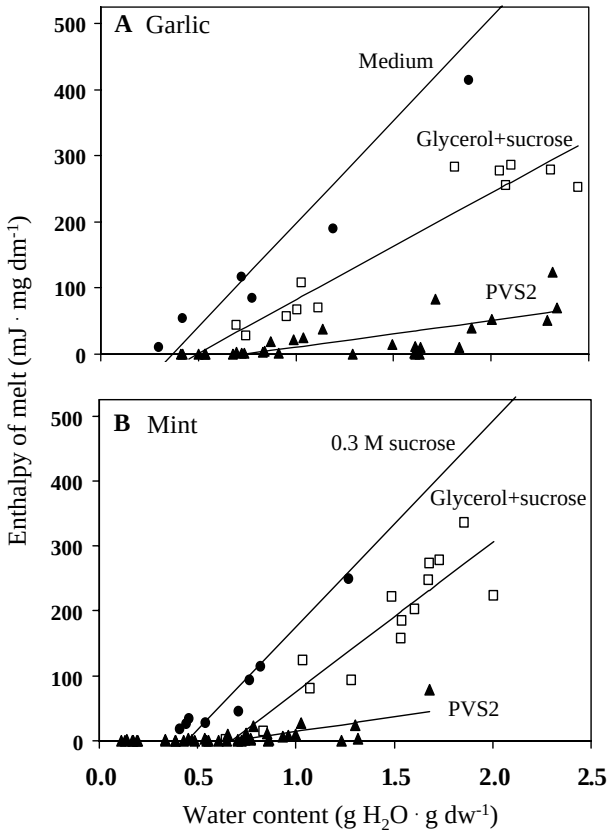

Fig. 6. The relationship between water content and enthalpy of melt in single garlic shoot tips (A) and sets of eight mint shoot tips (B). Shoot tips from medium or 0.3 M sucrose (solid circles), glycerol + sucrose (open squares) or PVS2 (solid triangles) for different lengths of time. The slope of the regression lines represents the melting enthalpy on a per mass of water basis for each treatment (shown in Table 1). The *x*-intercept is the water content at which there are no melting transitions observed (shown in Table 1).

Table 1
Enthalpy of melting transition per gram of water (as determined from slope of lines in Fig. 7) and shoot tip water content below which melting transitions are not observed (as determined from *x*-intercept of lines in Fig. 7)

Explant type	Treatment	Enthalpy of melt per gram water (J g H_2O^{-1})	Water content (g H_2O g dm^{-1})	r^2
Garlic	Medium	317 ± 18 a	0.38	0.98
	Glyc + suc	162 ± 16 b	0.49	0.91
	PVS2	40 ± 7 c	0.74	0.56
Mint	0.3 M suc	309 ± 11 a	0.43	0.99
	Glyc + suc	231 ± 30 b	0.67	0.83
	PVS2	45 ± 10 c	0.68	0.48

Regression coefficients (r^2) for lines in Fig. 7 are provided. Enthalpy of melting transition per gram of water is significantly different (denoted by letters) across treatments within a species.

reduced with PVS2 exposure and glass transitions became apparent (Figs. 1–4). However, PVS2 quenched first order transitions more than expected by dehydration alone (Figs. 6 and 7). Furthermore, the glass transitions observed in PVS2-treated shoot tips were atypical to current concepts of vitrification in biology. Generally, "good glass formers" are expected to be better protectants because they have higher glass transition temperatures (T_g), and T_g is expected to increase with dehydration as a result of the plasticizing effect of water [19]. The expected glassy behavior was exhibited in glycerol + sucrose treated mint shoot tips, in which T_g increased from −80 to about −40 °C for shoot tips containing 2 (Fig. 1D scan #2) and 0.84 g H_2O g dm^{-1} (Fig. 7D). The T_g for garlic shoot tips treated with glycerol

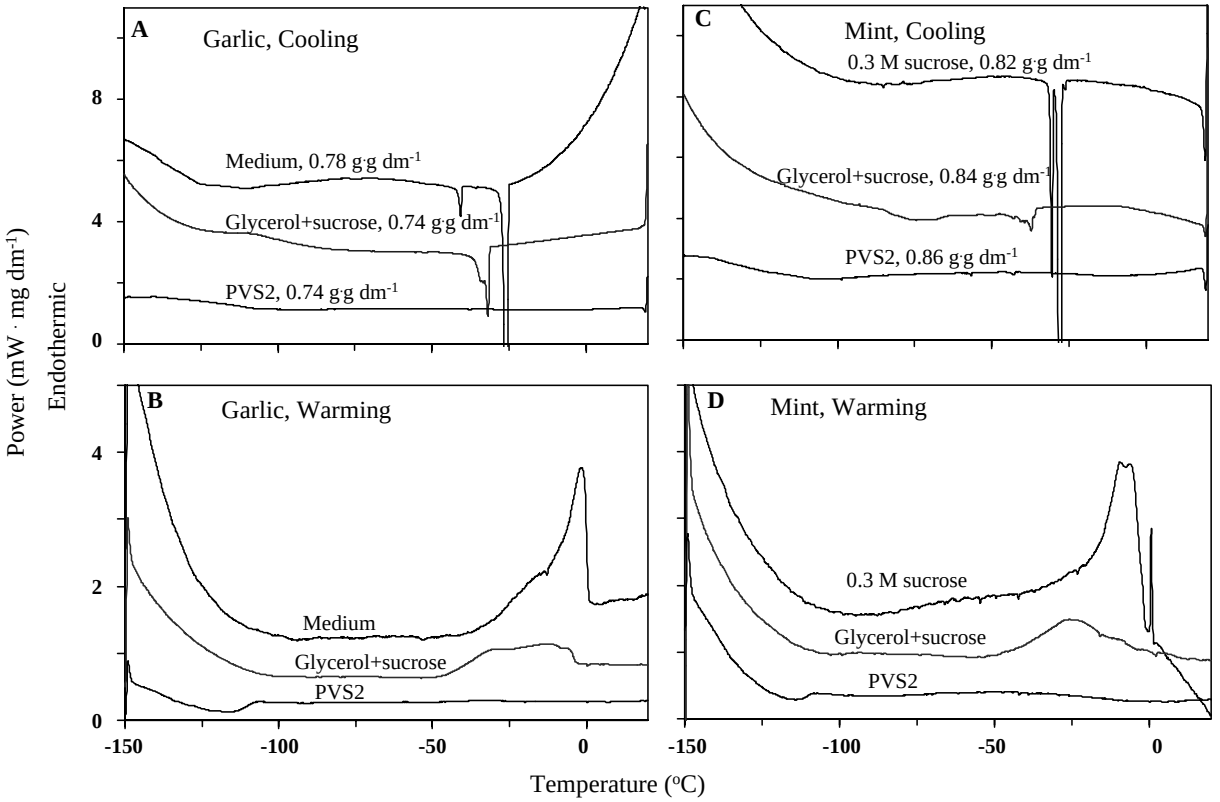

Fig. 7. DSC scans showing first order transitions that occur during garlic cooling (A) and warming (B) and mint cooling (C) and warming (D). Single garlic or sets of eight mint shoot tips were treated with either medium (or 0.3 M sucrose), 2 M glycerol + 0.6 M sucrose, or PVS2 and air dried to the same corrected water contents (as shown on the lines of the scans).

+sucrose also appears to increase with decreasing water content (compare Fig. 1B scan #2 with Fig. 7B) if one interprets the increase in power at −50 °C in Fig. 7B as a devitrification event. Expected glassy behavior was also exhibited in glycerol+sucrose treated mint shoot tips that resisted water loss under ambient conditions for at least two months (water contents were maintained at 0.5 g H_2O g dm^{-1} for 18 h (Fig. 8B) and 0.2 g H_2O g dm^{-1} for >2 months (data not shown)). Unexpected glassy behavior was exhibited in PVS2-treated shoot tips: T_g decreased to −115 °C (Figs. 3 and 4) and remained constant despite drying (Figs. 1, 3, and 7).

Our data indicate that PVS2 does not induce glass transitions at sub-zero temperatures near 0 °C and that glass transition temperatures and magnitudes do not reflect the degree of cryoprotection. Nonetheless, PVS2 has a remarkable ability to limit freezing of water. We interpret the apparent contradiction of preventing water freezing without forming glass as an indication that PVS2 imparts its effect in 'pre-vitrified' solutions. These solutions have sufficient mobility to permeate cells at 0 °C and allow water to be displaced. At lower temperatures, these cryoprotective solutions restrict molecular reorganization necessary to nucleate water and allow ice crystals to grow. This suggestion is supported by the >50 °C difference between glass melting and recrystallization that is observed in shoot tips receiving only short exposures to PVS2 (Fig. 3). It is not clear whether the limited crystallization is mediated by a reduction in mobility, typically viewed as the primary function of glasses, or a destabilization of crystalline structure. The mechanism of function is important for longevity in cryogenic storage, because reduced cellular viscosity will likely increase long-term survival [7,51]. Storage of preserved materials well below the glass transition temperature is advocated since molecules are mobile at T_g [7,9,10,51]. Our demonstration that T_g occurred at −115 °C in PVS2-treated shoot tips, the same temperature as that which occurs in PVS2 solution, supports the storage of cryopreserved shoot tips at liquid LN temperatures.

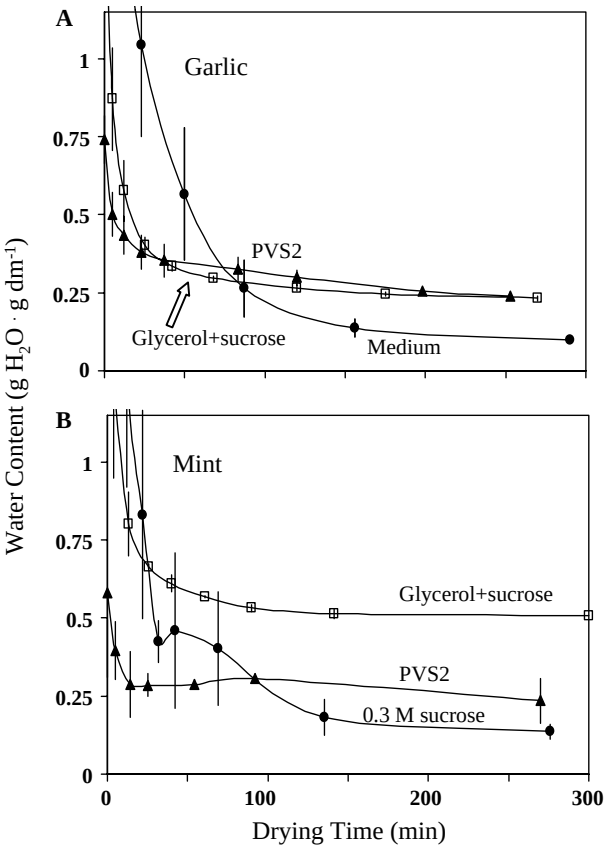

Fig. 8. Corrected water contents for single garlic shoot tips (A) or sets of eight mint shoot tips (B) after treatment with medium (or 0.3 M sucrose), 2 M glycerol + 0.6 M sucrose, or PVS2 and then air dried.

Garlic and mint shoot tips treated with PVS2 for 30 min, showed no indication of water freezing transitions in DSC experiments even though survival rates were 35 and 75%, respectively. This suggests that the mortality observed following cryoprotection and LN exposure was not a result of intracellular ice. More likely, shoot tips were damaged by the extreme desiccation allowed by PVS2 (Fig. 6), by toxicity from the components of PVS2 [50], or by the influx of water following LN exposure. The high water contents measured when cryoexposed shoot tips were returned to solid medium (Fig. 2D) may be symptomatic of reparable damage to membranes in many shoot tips or evidence of lethal damage in a subsample of shoot tips. Survival rates of cryoprotected mint and garlic shoot tips that were not exposed to LN were 95 and 82%, demonstrating some injury in garlic by the protection procedures. Data presented here and elsewhere (Volk and Caspersen, submitted) suggest that PVS2 does not cause massive cell shrinkage that is damaging to cells. However, PVS2 dries samples below the unfrozen water content (compare water contents in Fig. 5 and in Table 1), a moisture level believed to be critical to survival of desiccation sensitive organs [18,47,52].

Cryoprotection and recovery of plant shoot tips after LN exposure is associated with a reversible loss and readjustment of water within cells without major fluctuations in shoot tip fresh mass or cell size. When shoot tips are fully protected using PVS2, there is no evidence of water freezing transitions. However, evidence that PVS2 prevented ice formation by forming a glass is weak, and we suggest the protective mechanism is based on a partial restriction of molecular mobility and/or a disorganization of ice crystal structure.

Acknowledgments

We thank Lisa Hill, Kate Rotindo, and Jackie Harris for laboratory assistance.

References

[1] C.A. Angell, Liquid fragility and the glass transition in water and aqueous solutions, Chem. Rev. 102 (2002) 2627–2650.

[2] A. Baudot, L. Alger, P. Boutron, Glass-forming tendency in the system water–dimethyl sulfoxide, Cryobiology 40 (2000) 151–158.

[3] A. Baudot, V. Odagescu, Thermal properties of ethylene glycol aqueous solutions, Cryobiology 48 (2004) 283–294.

[4] E.E. Benson, B.M. Reed, R.M. Brennan, K.A. Clacher, D.A. Ross, Use of thermal analysis in the evaluation of cryopreservation protocols for *Ribes nigrum* L. germplasm, Cryo Letters 17 (1996) 347–362.

[5] W. Block, Water status and thermal analysis of alginate beads used in cryopreservation of plant germplasm, Cryobiology 47 (2003) 59–72.

[6] G. Bryant, K.L. Koster, J. Wolfe, Membrane behaviour in seeds and other systems at low water content: the various effects of solutes, Seed Sci. Res. 11 (2001) 17–25.

[7] J. Buitink, O. Leprince, Glass formation in plant anhydrobiotes: survival in the dry state, Cryobiology 48 (2004) 215–228.

[8] J. Buitink, C. Walters-Vertucci, F.A. Hoekstra, O. Leprince, Calorimetric properties of dehydrating pollen: analysis of a desiccation tolerant and an intolerant species, Plant Physiol. 111 (1996) 235–242.

[9] Z. Chang, J.G. Baust, Further inquiry into the cryobehavior of aqueous solutions of glycerol, Cryobiology 28 (1991) 268–278.

[10] Z.H. Chang, J.G. Baust, Physical aging of glassy state: DSC study of vitrified glycerol systems, Cryobiology 28 (1991) 87–95.

[11] J.H. Crowe, J.F. Carpenter, L.M. Crowe, The role of vitrification in anhydrobiosis, Annu. Rev. Physiol. 60 (1998) 73–103.

[12] J. Dereuddre, M. Kaminski, Applications of thermal analysis in cryopreservation of plant cells and organs, J. Thermal Anal. 38 (1992) 1965–1978.

[13] D. Dumet, W. Block, R. Worland, B.M. Reed, E.E. Benson, Profiling cryopreservation protocols for *Ribes ciliatum* using differential scanning calorimetry, Cryo Letters 21 (2000) 367–378.

[14] G.M. Fahy, The relevance of cryoprotectant "toxicity" to cryobiology, Cryobiology 23 (1986) 1–13.

[15] G.M. Fahy, D.I. Levy, S.E. Ali, Some emerging principles underlying the physical properties, biological actions, and utility of vitrification solutions, Cryobiology 24 (1987) 196–213.

[16] G.M. Fahy, D.R. MacFarlane, C.A. Angell, H.T. Meryman, Vitrification as an approach to cryopreservation, Cryobiology 21 (1984) 407–426.

[17] G.M. Fahy, T.H. Lilley, H. Linsdell, M.S. Douglas, H.T. Meryman, Cryoprotectant toxicity and cryoprotectant toxicity reduction: in search of molecular mechanisms, Cryobiology 27 (1990) 247–268.

[18] J.M. Farrant, C. Walters, Ultrastructural and biophysical changes in developing embryos of *Aesculus hippocastanum* L. in relation to the acquisition of tolerance to drying, Physiol. Plant 104 (1998) 513–524.

[19] F. Franks, Biophysics and Biochemistry at Low Temperatures, Cambridge University Press, Cambridge, UK, 1985.

[20] B.J. Fuller, Cryoprotectants: the essential antifreezes to protect life in the frozen state, Cryo Letters 25 (2004) 375–388.

[21] A.G. Hirsh, R.J. Williams, H.T. Meryman, A novel method of natural cryoprotection, Plant Physiol. 66 (1985) 40–45.

[22] H.-H. Kim, J.-B. Kim, H.-J. Baek, E.-G. Cho, Y.-A. Chae, F. Engelmann, Evolution of DMSO concentration in garlic shoot tips during a vitrification procedure, Cryo Letters (2004) 91–100.

[23] H.-H. Kim, J.-W. Yoon, J.-B. Kim, F. Engelmann, E.-G. Cho, Thermal analysis of garlic shoot tips during a vitrification procedure, Cryo Letters 26 (2005) 33–44.

[24] R. Langis, P.L. Steponkus, Vitrification of isolated rye protoplasts: protection against dehydration injury by ethylene glycol, Cryo Letters 12 (1991) 107–112.

[25] D. Martinez, M.A. Revilla, A. Espina, J.R. Garcia, Differential scanning calorimetry applied to the storage at ultra low temperatures of olive and hop in vitro grown shoot-tips, Thermochim. Acta 349 (2000) 147–151.

[26] D. Martinez, M.A. Revilla, A. Espina, E. Jaimez, J.R. Garcia, Survival cryopreservation of hop shoot tips monitored by differential scanning calorimetry, Thermochim. Acta 317 (1998) 91–94.

[27] P. Mazur, Principles of cryobiology, in: B.J. Fuller, N. Lane, E.E. Benson (Eds.), Life in the Frozen State, CRC Press, 2004, pp. 3–65.

[28] H.T. Meryman, Freezing injury and its prevention in living cells, Annu. Rev. Biophys. Bioeng. 3 (1974) 341–363.

[29] A.S. Meyer Jr., C.M. Boyd, Determination of water by titration with coulometrically generated Karl Fischer Reagent, Anal. Chem. 31 (1959) 215–219.

[30] T. Murashige, F. Skoog, A revised medium for rapid growth and bio assays with tobacco tissue cultures, Physiol. Plant 15 (1962) 473–497.

[31] S.P. Myers, P.L. Steponkus, Permeation of dimethyl sulfoxide into rye protoplasts during freezing of the suspending medium, Cryo Letters 7 (1986) 41–54.

[32] T. Niino, A. Sakai, H. Yakuwa, K. Nojiri, Cryopreservation of in vitro-grown shoot tips of apple and pear by vitrification, Plant Cell Tiss. Org. Culture 28 (1992) 261–266.

[33] S. Nishizawa, A. Sakai, Y. Amano, T. Matsuzawa, Cryopreservation of asparagus (*Asparagus officinalis* L.) embryogenic suspension cells and subsequent plant regeneration by vitrification, Plant Sci. 91 (1993) 67–73.

[34] C. Polge, A.U. Smith, A.S. Parkes, Revival of spermatozoa after vitrification and dehydration at low temperatures, Nature 164 (1949) 666.

[35] W.F. Rall, Factors affecting the survival of mouse embryos cryopreserved by vitrification, Cryobiology 24 (1987) 387–402.

[36] A. Sakai, Development of cryopreservation techniques, in: F. Engelmann, H. Takagi, (Eds.), Cryopreservation of Tropical Plant Germplasm. Current research progress and application. Japan International Research Center for Agricultural Sciences, Tsukuba, Japan/International Plant Genetic Resources Institute, Rome, Italy, 2000, pp. 1–7.

[37] A. Sakai, S. Kobayashi, I. Oiyama, Cryopreservation of nucellar cells of navel orange (*Citrus sinensis* Osb. var. *brasiliensis* Tanaka) by vitrification, Plant Cell Rep. 9 (1990) 30–33.

[38] A. Sakai, S. Kobayashi, I. Oiyama, Survival by vitrification of nucellar cells of navel orange (*Citrus sinensis* var. *brasiliensis* Tanaka) cooled to −196 °C, J. Plant Physiol. 137 (1991) 465–470.

[39] G. Sherlock, W. Block, E.E. Benson, Thermal analysis of the plant encapsulation–dehydration cryopreservation protocol using silica gel as the desiccant, Cryo Letters 26 (2005) 45–54.

[40] P.L. Steponkus, R. Langis, S. Fujikawa, Cryopreservation of plant tissues by vitrification, in: P.L. Steponkus (Ed.), Advances in Low-Temperature Biology, JAI Press, Greenwich, CT, 1992, pp. 1–61.
[41] P.L. Steponkus, M. Uemura, M.S. Webb, Freeze-induced destabilization of cellular membranes and lipid bilayers, in: E.A. Disalvo, S.A. Simon (Eds.), Permeability and Stability of Lipid Bilayers, CRC Press, Boca Raton, 1995, pp. 77–104.
[42] L.E. Towill, Cryopreservation of isolated mint shoot tips by vitrification, Plant Cell Rep. 9 (1990) 178–180.
[43] A. Uragami, A. Sakai, M. Nagai, T. Takahashi, Survival of cultured-cells and somatic embryos of *Asparagus officinalis* cryopreserved by vitrification, Plant Cell Rep. 9 (1989) 418–421.
[44] C.W. Vertucci, Effects of cooling rate on seeds exposed to liquid nitrogen temperatures, Plant Physiol. 90 (1989) 1478–1485.
[45] C.W. Vertucci, Relationship between thermal transitions and freezing injury in pea and soybean seeds, Plant Physiol. 90 (1989) 1121–1128.
[46] C.W. Vertucci, P. Berjak, N.W. Pammenter, J. Crane, Cryopreservation of embryonic axes of an homoiohydrous (recalcitrant) seed in relation to calorimetric properties of tissue water, Cryo Letters 12 (1991) 339–350.
[47] C.W. Vertucci, C. Stushnoff, The state of water in acclimating vegetative buds of apple and *Amelanchier* and its relationship to winter hardiness, Physiol. Plant 86 (1992) 503–511.
[48] G.M. Volk, A. Henk, C. Richards, Genetic diversity among US garlic clones as detected using AFLP methods, J. Am. Soc. Hort. Sci. 129 (2004) 559–569.
[49] G.M. Volk, N. Maness, K. Rotindo, Cryopreservation of garlic (*Allium sativum L.*) using plant vitrification solution 2, Cryo Letters 25 (2004) 219–226.
[50] G.M. Volk, J.L. Harris, K.E. Rotindo, Survival of mint shoot tips after exposure to cryoprotectant solution components, Cryobiology (2006), in press.
[51] C. Walters, Temperature-dependency of molecular mobility in preserved seeds, Biophys. J. 86 (2004) 1253–1258.
[52] J. Wesley-Smith, C.W. Vertucci, P. Berjak, N.W. Pammenter, J. Crane, Cryopreservation of desiccation-sensitive axes of *Camellia sinensis* in relation to dehydration, freezing rate, and the thermal properties of tissue water, J. Plant Physiol. 140 (1992) 596–604.
[53] J. Wesley-Smith, C. Walters, N.W. Pammenter, P. Berjak, Interactions of water content, rapid (non-equilibrium) cooling to −196 °C and survival of embryonic axes of *Aesculus hippocastanum* seeds, Cryobiology 42 (2001) 196–206.
[54] J. Wolfe, G. Bryant, K.L. Koster, What is 'unfreezable water', how unfreezable is it and how much is there? Cryo Letters 23 (2002) 157–166.